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Identification and bioactivity evaluation of two novel temporins from the skin secretion of the European edible frog, *Pelophylax kl.esculentus*

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Abstract

Two novel members of the temporin family, named temporin-1Ee (FLPVIAGVLSKLF_{amide}) and temporin-1Re (FLPGLLAGLL_{amide}), whose biosynthetic precursor structures were deduced from clones obtained from skin secretion-derived cDNA libraries of the European edible frog, *Pelophylax kl. esculentus*, were obtained by 'shotgun' cloning. Deduction of the molecular masses of each mature processed peptide from respective cloned cDNAs was used to locate respective molecules in reverse-phase HPLC fractions of secretion. Temporin-1Ee (MIC=10 μ M) and temporin-1Re (MIC=60 μ M) were both found to be active against Gram-positive *Staphylococcus aureus*, but retaining a weak haemolytic activity. Single-site substitutions can dramatically change the spectrum of activity of a given temporin. Compared with temporine-1Ec, just one chemically-conservative substitution (Val8 instead of Leu8), temporin-1Ee bearing a net charge of +2 displays broad-spectrum activity with particularly high potency on the clinically relevant Gram-negative strains, *Escherichia coli* (MIC=40 μ M). These factors bode well for translating temporins to be potential anti-infective drug candidates.

Keywords:Amphibian; Antimicrobial Peptide; Temporins;Cloning

Introduction

Temporins, containing 10-13 amino acid residues, are considered to be among the smallest AMPs found in Nature. They were initially described as the Vespa-like peptides, due to their sequence similarities with chemotactic and histamine-releasing peptides isolated from the venom of wasps of the genus *Vespa*, and were first identified in the skins of the European edible frog, *Pelophylax kl. esculentus* and the Asian frog, *Rana erythraea*. Afterwards, a family of 10, structurally-related peptides with antibacterial and antifungal properties were isolated from the electrically-stimulated skin secretions of the European common red frog, *Rana temporaria*, and called temporins A to L, in reflection of their source (1). Compared with other ranid frog skin AMPs that have a C-terminal heptapeptide ring constructed by a disulphide bridge, the temporins are C-terminally α -amidated and stabilised by helix-forming residues such as leucine, alanine and lysine. Due to their basic residues, temporins usually carry a net charge ranging from 0 to +4 at neutral pH and have a potent activity against Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA) with little toxicity to mammalian cells (2,3). However, since temporin L is the first member of this family isolated which exhibit broad spectra of activity against both Gram-positive and Gram-negative bacteria (Giacometti et al 2006), a few new members of the temporin family, such as temporin-Sha, temporin-T1 and temporin-1DRa, have been found to display similar antimicrobial activities to temporin L (4, 5).

Naturally occurring AMPs differ dramatically in various interrelated peptide parameters, including size, net charge, hydrophobicity, amphipathicity, degree of structure formation and flexibility (6-8), thus the selectivity and mechanism of antimicrobial activities remain unknown. It is well accepted that differences in lipid composition of membranes among microorganisms play key roles in the selectivity of AMPs toward microbial cells (2), and

although the mechanism of action of temporins have been only scarcely evaluated, most are supposed to have a classical membrane perturbing activity through a barrel-stave mechanism of action. In contrast, temporin L is presumed to insert into bacterial cell membranes through the formation of pore-like openings and interact with essential anionic targets inside the cells (1-3, 9-10). Temporins as short chain peptides of simple structure are considered to be good templates for new drug design.

Herein, two novel antimicrobial peptides have been identified in the skin secretion of the European Edible frog, *Pelophylax kl. esculentus*, through initial molecular cloning of skin secretion-derived cDNAs. Deduced peptides were subsequently identified in fractions of skin secretion, chemically-synthesised and subjected to functional analyses. Sequence characterisation of the mature peptides demonstrated that they belonged to the temporin family found predominantly in the skin secretions of ranid frogs. Thus, these two peptides were named temporin-1Ee and temporin-1Re. Temporin-1Ee, which is an analogue of temporin-1Ec, has broad-spectrum antimicrobial activity at low micromolar concentrations. Temporin-1Re, with a net charge of +1, is weakly active against the Gram-positive bacterium, *S. aureus*, but is devoid of activity against Gram-negative bacteria and yeast.

Materials and methods

Preparation of *P. kl.esculentus* skin secretion

P.kl.esculentus ($n = 30$) were obtained from a commercial source (Le QuerryCellier, Notre Dame de Monts, France) and had been captive bred. The frogs were adults on receipt and were settled into their new surroundings for 3 months prior to secretion harvesting. They were maintained in our purpose-designed amphibian facility at 15–20 °C under a 12 h/12 h light/dark cycle and fed multivitamin-loaded crickets three times per week. Skin secretion was obtained from dorsal skin by mild transdermal electrical stimulation (5V; 3 ms pulses) for 30 s. The skin secretion was washed from the skin using deionized water, snap-frozen in liquid nitrogen, and lyophilized. Lyophilizate was stored at –20 °C prior to analysis.

“Shotgun” cloning of *Pelophylax kl. esculentus* skin secretion-derived cDNA

Five milligrams of lyophilised skin secretion were dissolved in 1ml lysis/binding buffer (Dynal, UK) to stabilise mRNA. Polyadenylated mRNA was trapped and isolated by means of magnetic oligo-dTDynabeads as described by the manufacturer (Dynal, UK). The trapped template polyadenylated mRNA was employed to manufacture a cDNA library using reverse transcriptase and a sample of this library was subjected to 5'- and 3'-rapid amplification of cDNA ends (RACE) procedures to obtain full-length antimicrobial peptide precursor nucleic acid sequence data using a SMART-RACE kit (Clontech UK) following manufacturer's instructions. Briefly, the 5'-RACE reactions employed the forward primer (5'-ATGTTTCACCATGAAGAAATC-3') and the reverse primer (5'-CTATCCCACATCAGGAGACTTTCC-3') were designed according to the nucleotide sequence of temporin precursor cDNAs obtained from other ranid frogs. The 5'-RACE reactions were purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Reverse phase HPLC fractionation of skin secretion

A further 1mg sample of lyophilised skin secretion was dissolved in 0.5ml of 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and clarified of microparticulates by centrifugation. The supernatant was then subjected to HPLC using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/29.95/70.0 (v/v/v) TFA/water/acetonitrile in 240min at a flow rate of 1 ml/min. A Cecil Adept Binary HPLC system (Adept Technology, Inc. USA), fitted with an analytical column (a Jupiter 00G4052 semi-preparative C-5 column 25x1cm, Phenomenex, U.K.) was employed and this was interfaced with a Thermo Fisher Scientific LCQ Fleet electrospray ion-trap mass spectrometer. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass spectrometer source and 90% directed towards a fraction collector. Dead volume between column and fraction collector was minimal (20 μ l). The molecular masses of polypeptides in each chromatographic fraction were further analysed using matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as $\pm 0.1\%$.

Peptide synthesis and purification

Following unequivocal establishment of the primary structure of natural temporin-1Ee and temporin-1Re, replicates were synthesised by solid-phase Fmoc chemistry using a PS3 automated peptide synthesiser (Protein Technologies, Tucson, AZ, USA). When the synthesis cycles were completed, the peptides were cleaved from the resins using 95/2.5/2.5 (v/v/v) TFA/TIPS/water for 6h, precipitated in ether over the next 24 h, washed exhaustively in six changes of ether and then allowed to completely dry over a further 24h. The peptides were then dissolved in a minimal quantity of 0.05/99.5, v/v, TFA/water, snap frozen in liquid

nitrogen and lyophilised. Degree of purity and authentication of structures of the synthetic peptides were determined using MALDI-TOF MS as previously described.

Antimicrobial assays

Antimicrobial activities of synthetic temporin-1Ee and temporin-1Re were assessed by determination of minimal inhibitory concentrations (MICs) using a standard Gram-positive bacterium *S. aureus*—NCTC 10788), a standard Gram-negative bacterium (*Escherichia coli*—NCTC 10418) and a standard pathogenic yeast (*Candida albicans* NCPF 1467). Temporin-1Ee and temporin-1Re were tested within the concentration range of 160–2.5 μ M and were initially dissolved as a stock solution of 200 μ M in sodium phosphate-buffered saline, pH 7.2, and subsequently diluted in Mueller–Hinton broth (MHB). Peptide concentrations in the range stated, were inoculated with microorganism cultures (105 colony forming units (CFU)/ml), and placed into 96-well microtiter cell culture plates. Plates were incubated for 18 h at 37°C in a humidified atmosphere. Following this, the growth of bacteria/yeast was determined by means of measuring optical density (OD) at λ =550nm by an ELISA plate reader (BioliseBioTek EL808). Minimal inhibitory concentrations (MICs) were defined as the lowest concentration at which no growth was detectable.

Results

Molecular cloning of the A biosynthetic precursor

Two different cDNAs, each encoding a novel peptide, named temporin-1Ee and temporin-1Re, respectively, were consistently and repeatedly cloned from the library during RACE-PCR experiments (Figure1). Such experiments used *R. esculenta* skin mRNA as a template and specific oligonucleotides, designed to a conserved region (Signal peptide and 3'-UTR) of previously cloned preprotemporincDNAs from other ranid species. The cDNAs amplified were separated on agarose gels then isolated with the cloning vector which was transformed into host cells. The white and drug resistant bacterial colonies were chosen by random and subjected to nucleotide sequence analysis. Alignment of temporin-1Ee and -1Re nucleotide sequences and open-reading frame amino acid sequences, using the AlignX programme of the Vector NTI Bioinformatics suite (Informax), revealed a very high degree of primary structural similarity of both nucleic acid and amino acid sequence between the temporin-1Ee and -1Re (Figure 2). An NCBI BLAST search found that the novel temporin-1Ee showed at least 92% sequence identity with temporin-1Ec and peptide A1 and peptide B9 obtained from *R. esculenta*, previously (Table 2). In the precursor sequence, the first 22 amino acid residues encoded a putative signal peptide at the N-terminal followed by an acidic amino acid residue rich spacer peptide consisting of 21 amino acids for temporin-1Ee and 22 amino acids for temporin-1Re with a pair of classical basic residue -Lys-Arg- (-K-R-) propeptide convertase cleavage sites at its C-terminus. A single copy of a mature temporin sequence was found at the C-terminus just upstream of a Gly residue that serves as an amide donor for the C-terminal residue of the temporin. The nucleotide sequence of the temporin-1Ee and temporine-1Re precursors from the skin secretion of the European edible frog, *Pelophylax kl.esculentus*, have been deposited in the EMBL Nucleotide Sequence Database under the accession code KT437658 and KT437659.

Identification and structural analysis of mature temporin-1Ee and temporin-1Re in skin secretion fractions and subsequent chemical synthesis

The two novel temporins (temporin-1Ee: FLPVIAGVLSKLF-amide and temporin-1Re: FLPGLLAGLL-amide), deduced from cloned skin cDNAs, with computed molecular mass of 1402.08Da and 1012.32Da, respectively, were identified in HPLC fraction numbers 164 and 143, respectively (Figures 3 and 4). Both temporins were successfully obtained by the solid-phase chemical synthesis. The reverse phase HPLC chromatograms obtained from each synthesis following cleavage from the resin and deprotection, showed major peaks in each case representing approximately 95% of the synthetic mixture and these were subjected to MALDI-TOF mass analysis. In each case, molecular ions consistent with predicted molecular masses of respective novel temporins were observed indicating successful syntheses and confirming a high degree of purity (>95%) of the expected products.

Antimicrobial/hemolytic activity of temporin-1Ee and temporin-1Re

The abilities of temporin-1Ee and temporin-1Re peptides to inhibit the growth of the tested microorganisms employed are compared in Table 3. MICs obtained against *S. aureus* were 10 μ M for temporin-1Ee and 60 μ M for temporin-1Re. Moreover, temporin-1Ee displayed an inhibitory effect on the growth of the Gram-negative bacterium, *E. coli*, MIC was 40 μ M, whereas temporin-1Re exhibit no effect. Both temporins were ineffective in inhibiting the growth of the yeast, *C. albicans*, at concentrations up to and including 160 μ M. Moreover, temporin-1Ee was found to possess no haemolytic activity at the MIC (10 μ M) determined for *S. aureus*. This experiment was repeated five times.

Discussion

The AMPs stored in the glands of frog skin display a complicated array of typical peptide physico-chemical attributes related to size, charge, hydrophobicity, degree of secondary structure formation and amphipathicity. Temporins have been intensely-studied due to their potent activity against Gram-positive bacteria, including clinical isolates of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* (9,12). However, due to their small size and low charge, the antimicrobial activity mechanisms of temporins are still unclear. The most recent research has provided some new insights to explain the mechanism of action of the temporins. Take temporin-L as an example. Temporin-L is a 13-mer peptide in which a tail-to-tail dimerisation can occur that then suits the barrel-stave model, binding to the vesicles surface, and undergoing a rapid local aggregation that causes a reorientation. Membrane insertion steps can only occur after this oligomerisation (12-14). We presume that temporin-1Ee, which has the same chain length as temporin-L and also possesses antimicrobial activity against both Gram-negative and Gram-positive bacteria, may act via a similar mechanism in which the carpet model could explain this temporin's antimicrobial activity but the barrel-stave model could explain the haemolytic effect (1-3, 15).

Hence the variation in net charges (0 to +3), chain length (generally 10-14 residues), and amino acid sequences could influence the efficacy and selectivity of temporins against pathogens. Most members of the temporin family carry a low net charge of 0 or +1 at physiological pH and their chain lengths are restricted and small. Within the temporins, the higher the net positive charge (+2 or +3), the more potent they are in killing bacteria. Temporin-1Ee, with a net charge of +2, is more potent (MIC 10 μ M) than temporin-1Re (MIC 60 μ M), with a net charge of +1, in killing the model Gram-positive bacterium, *Staphylococcus aureus* (NCTC 10788). In contrast, due to most of the natural

temporins having a weak charge and small size, they generally have little or no effects on Gram-negative bacteria, fungi and yeasts (16-20). However, one of the prototype temporins, temporin L (13 residues, net charge +3), was found to exhibit broad-spectrum activity against many microorganisms, including Gram-positive bacteria, Gram-negative bacteria and yeast, even cancer cells and human erythrocytes. Many additional temporins subsequently discovered exhibit broad-spectrum antimicrobial activity, examples being temporin-1Sa (11) and temporin-1DRa (21-22). Temporin-1Ee, with a net charge of +2, has efficacy and a high degree of activity against the model Gram-positive bacterium, *S. aureus*, with an MIC close to that of temporin-1Ec (MIC 8 μ M) (23). However temporin-1Ee, which has an amino acid sequence quite similar to that of temporin-1Ec with just one chemically-conservative substitution (Val8 instead of Leu8 – Table 2.), is able to inhibit the growth of the model Gram-negative bacterium, *Escherichia coli*. Thus even conservative, single-site substitutions can dramatically change the spectrum of activity of a given temporin. The temporins and their analogues are thus well accepted candidates for the design of new antimicrobial peptides for reasons such as above, where small changes in amino acid sequence can result in dramatic improvements in antimicrobial activity but retaining a weak haemolytic activity (24-27). These factors bode well for translating temporins into clinically useful anti-infective agents to combat antibiotic-resistant pathogens.

Acknowledgments

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data deposition footnotes

The nucleotide sequence of the temporin-1Ee and temporine-1Re precursors from the skin secretion of the European edible frog, *Pelophylax kl.esculentus*, have been deposited in the EMBL Nucleotide Sequence Database under the accession code KT437658 and KT437659.

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Legends to Figures

Figure 1. Nucleotide sequences of cloned cDNAs encoding the biosynthetic precursors of temporin-1Ee (A) and -1Re (B) and translated amino acid sequences of their open-reading frames. Putative signal peptides are double-underlined, mature peptides are single-underlined and the stop codons are indicated by asterisks. The G residue (*italics*) at the C-terminal of the mature peptide sequence serves as an amide donor after removal of the Lys residue (*italics*) with a carboxypeptidase. Residues G or GK (*italics*) at the end of the mature peptide sequence serve for C-terminal amidation.

Figure 2. (A) Alignment of full-length nucleotide sequences of clones encoding the precursors of temporin-1Ee and temporin-1Re. DNA sequences were copied in FASTA format, which were then subjected to multiple sequence alignment by the CLUSTALW program. (B) Alignment of open-reading frame amino acid sequences of preprottemporin-1Ee and preprottemporin-1Re. The conserved residues are shaded. Amino acid sequences were copied in FASTA format, which were then subjected to multiple sequence alignment by the CLUSTALW program.

Figure 3. Reverse phase HPLC chromatogram of the skin secretion from *Rana esculenta* with arrows indicating elution positions/retention times of the two novel antimicrobial peptides, temporin-1Re and temporin-1Ee. The Y-axis indicates absorbance units at $\lambda=214\text{nm}$.

Figure 4. (A) MALDI-TOF mass spectrum showing major peptide molecular ion in reverse phase HPLC fraction ~ 164 of *Pelophylax kl. esculentus* skin secretion corresponding in mass to temporin-1Ee (1402.08Da). (B) MALDI-TOF mass spectrum of peptide molecular ion in reverse phase HPLC fraction ~ 143 of skin extract corresponding to temporin-1Re (1012.32Da).

Figure 1

(A)

M F T M K K S L L L L F F L G T I
1 ATGTTACCA TGAAGAAATC CCTGTTACTC CTTTCTTCC TTGGGACCAT
TACAAGTGGT ACTTCTTTAG GGACAATGAG GAAAAGAAGG AACCCGTGTA
N L S L C E Q E R D A E E E R R
51 CAACTTATCT CTCTGTGAGC AAGAGAGAGA TGCTGAGGAA GAAAGAAGAG
GTTGAATAGA GAGACACTCG TTCTCTCTCT ACGACTCCTT CTTTCTTCTC
D D P E E R D V E M E K R F L P V
101 ACGATCCAGA AGAAAGGGAT GTTGAAATGG AAAAACGATT TTTACCAGTC
TGCTAGGTCT TCTTTCCCTA CAACTTTACC TTTTGTCTAA AAATGGTCAG
I A G V L S K L F G K *
151 ATTGCTGGAG TTTTGAGTAA ACTGTTCTGGC AAATAACCCA AAAATGTTGA
TAACGACCTC AAAACTCATT TGACAAGCCG TTTATTGGGT TTTTACAACT
201 AACTTTGGAA ATAGAATTGG AAATCATCTG ATGTGGAATA TCATTTAGCT
TTGAAACCTT TATCTTAACC TTTAGTAGAC TACACCTTAT AGTAAATCGA
251 AAATGCACAA CAGATGTCTT ATAAAAAAT AAAGATATCA CATATAAAAA
TTTACGTGTT GTCTACAGAA TATTTTTTTTA TTTCTATAGT GTATATTTTT
301 AAAAAAAAAA AAAAAAAAAA AAAAA
TTTTTTTTTT TTTTTTTTTT TTTTT

(B)

M F T M K K S L L L L F F L G T I
1 ATGTTACCA TGAAGAAATC CCTGTTACTC CTTTTTTTCC TTGGAACCAT
TACAAGTGGT ACTTCTTTAG GGACAATGAG GAAAAAAGG AACCTTGTA
N L S L C E E E R D T D E E E R
51 CAACTTATCC CTTTGTGAGG AAGAGAGAGA TACTGATGAG GAAGAAAGAA
GTTGAATAGG GAAACACTCC TTCTCTCTCT ATGACTACTC CTTCTTTCTT
R D E P D E M D V E V E K R F L P
101 GAGATGAGCC AGATGAAATG GATGTTGAAG TGGAAAAACG ATTTTACCA
CTCTACTCGG TCTACTTTAC CTACAACCTC ACCTTTTTGC TAAAAATGGT
G L L A G L L G K *
151 GGTCTGCTTG CTGGTTTGTG GGGAAAGTAA CCAAAAATGT TGAAACTTTG
CCAGACGAAC GACCAAACAA CCCTTTCATT GGTTTTTACA ACTTTGAAAC
201 GAAATGGAAA AGGAAATCAT CTGATGTGGA ATATCATTTA GCTAAATGCT
CTTTACCTTT TCCTTTAGTA GACTACACCT TATAGTAAAT CGATTTACGA
251 TAACAGATGT CTTTATAAAA AATAAATAAA TATGTCGCAT AAGAAAGGCC
ATTGCTCTACA GAAATATTTT TTATTTATTT ATACAGCGTA TTCTTTCCGG
301 CAAAAAATAA AAAACCCAAA AAAAAAATTT TTTAAAGAAA AAA
GTTTTTTATT TTTTGGGTTT TTTTTTTAAA AATTTTCTTT TTT

Figure 2

(A)

		1	50
temporin-1Ee	(1)	ATGTTCCACCATGAAGAAATCCCTGTTACTCCTTTTCTTCCCTGGGACCAT	
temporin-1Re	(1)	ATGTTCCACCATGAAGAAATCCCTGTTACTCCTTTTCTTCCCTGGGAACCAT	
		51	100
temporin-1Ee	(51)	CAACTTATCTCTCTGTGAGCAAGAGAGAGATGCTGA---GGAAGAAAGAA	
temporin-1Re	(51)	CAACTTATCCCTTTGTGAGGAAGAGAGAGATACTGATGAGGAAGAAAGAA	
		101	150
temporin-1Ee	(98)	GAGACGATCCAGAAGAAAGGGATGTTGAAATGGAAAAACGATTTTACCA	
temporin-1Re	(101)	GAGATGAGCCAGATGAAATGGATGTTGAAATGGAAAAACGATTTTACCA	
		151	200
temporin-1Ee	(148)	GTCATTGCTGGAGTTTGTAGTAACTGTT CGGCAAATAACCCAAAAATGT	
temporin-1Re	(151)	GGTCTGCTTGCTGGTT-----TGTTGGGAAAGTAACC-AAAAATGT	
		201	250
temporin-1Ee	(198)	TGAAACTTTGGAAATAGAATTGGAAATCATCTGATGTGGAATATCATTTA	
temporin-1Re	(191)	TGAAACTTTGGAAATGGAAAAGGAAATCATCTGATGTGGAATATCATTTA	
		251	300
temporin-1Ee	(248)	GCTAAATGCACAAACAGATGTCTT-ATAAAAA---ATAAAGATATCACAT	
temporin-1Re	(241)	GCTAAATGCTTAACAGATGTCTTTATAAAAAATAAATAAATATGTCGCAT	
		301	350
temporin-1Ee	(294)	ATAAAA-----AAAAAAAAAAAAAAAAAAAAAAAA-----	
temporin-1Re	(291)	AAGAAAGGCCCAAAAAATAAAAAACCCAAAAAAAAAATTTTAAAAAGAA	
		351	
temporin-1Ee	(326)	---	
temporin-1Re	(341)	AAA	

(B)

temporin-1Ee	(1)	MFTMKKS LLLLFLGTLNLSLCEQERDAEEE-RRDDPEERDVEMEKRFLP
temporin-1Re	(1)	MFTMKKS LLLLFLGTLNLSLCEEERDADEEERRDEPDMDVEVEKRFLP
temporin-1Ee	(50)	-VIAGVLSKLF GK
temporin-1Re	(51)	GLLAGLLGK----

Figure 3

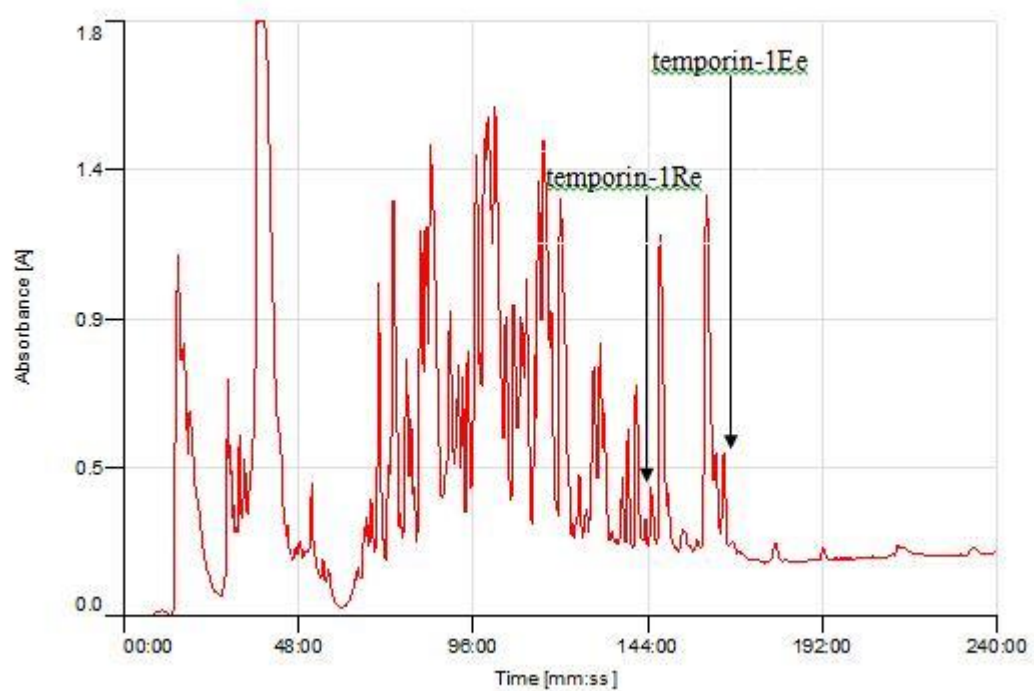


Figure 4

(A)



(B)

